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13. ABSTRACT (Maximum 200 Words)

In Year 1 of the project we have completed Task 1 (as per approved Statement of Work) by generating a panel of plasmid vectors for the expression of a series of modified adenovirus fiber genes. These genes encode genetic fusions of the adenovirus fiber protein, which has been stripped off its native receptor binding site by mutagenesis, with the previously identified peptides that are specific for the prostate tumor vasculature. The resultant proteins have been transiently expressed in cultured human cells and characterized by Western blot analysis.

As a result of this work, we have identified those fiber-peptide fusion proteins that retain the trimeric structure of native adenovirus fiber protein and, thus, can be incorporated into the capsid of Ad vector for tumor targeting.

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INTRODUCTION:

The scope of the proposed work is to develop novel Ad vectors, which will be targeted to vasculature of prostate tumors via genetic incorporation into their capsid of the recently identified endothelium-specific peptides. Additionally, tumor selectivity of these vectors is to be further improved by limiting the expression of the therapeutic transgene carried by the vectors, to endothelial cells of tumor vasculature. These double-targeted viruses are to be able to selectively infect blood vessels within the tumors and destroy the blood supply to tumors by locally expressing "suicide" transgene.

REPORT BODY:

As per the Statement of Work presented in the original project proposal, in Year 1 the following work was to be accomplished:

Task 1. Generation of recombinant Ad fiber proteins targeted to prostate vasculature endothelium (Months 1-9).

a. *Construction and validation of plasmid vectors incorporating modified Ad fiber genes for expression of the fibers in mammalian cells (Months 1-6).* Oligonucleotide duplexes encoding the peptides specific for prostate vasculature endothelium will be incorporated into the Ad5 fiber gene contained in $pVS_{HI}Bae$ and $pVS_{C}Bae$ mammalian expression vectors. The ligand-coding sequences will be incorporated into the regions of the gene corresponding to either the carboxy terminus or the HI loop of the fiber. The sequences immediately adjacent to targeting peptides will be varied in an attempt to facilitate optimal presentation of ligands.

b. *Transient expression of modified fiber genes in 293 cells and structural analysis of the expressed proteins (Months 7-9).* Newly derived expression vectors will be used for transfection of 293T cells to direct transient expression of modified fibers. The level of the fibers' expression and their capacity to form trimers will be assessed by Western blot analysis with anti-fiber antibody.

Task 2. Assessment of the receptor-binding capability of the modified fiber proteins (Months 9-12).

a. ELISA-based studies of the capacity of the proteins produced in Task 1 to bind to recombinant form of cognate cellular receptor (Months 9-12). Recombinant fibers transiently expressed in 293T cells will be used for ELISA studies using purified IL-11Rα. Binding of the fibers to IL-11Rα will be detected by using anti-fiber antibodies. The efficiency of this binding will be predictive of subsequent success in using these fibers for Ad targeting to IL-11Rα.

Description of work:

Task 1.

1. Modification of the fiber-expressing plasmid vectors. According to this plan, the work began with the construction of plasmid vectors for subsequent expression of the candidate fiber genes, which are to be designed in the project, in eukaryotic cells. To this end, we first modified the plasmids of the pVS series (3, 6) that contain Ad5 fiber genes with the modified carboxy terminus or the HI loop region. The purpose of these additional modifications was to mutate the fiber protein (FΔ2) such that it would no longer bind to the known Ad5 fiber receptors – CAR (5, 10) and HSG (9). As a result, these mutated FΔ2 proteins would be an ideal platform for the incorporation of tumor-specific targeting peptides, thereby yielding *truly targeted* fibers. This was accomplish by site-directed, PCR-based mutagenesis procedure that replaced the HSG-binding *KKTK* motif in the Ad5 fiber shaft domain with a *GAGA* tetrapepdide (9), and also deleted the *TYAT* sequence within the fiber knob domain (8).

Therefore, the plasmid vectors pVSΔ2.Fc.BaeI, pVSΔ2.PB10.BaeI, and pVSΔ2.PB40.BaeI were designed. pVSΔ.2Fc.BaeI was designed to make the fiber genes with targeting ligands positioned at the carboxy terminus of the fiber, whereas pVSΔ2.PB10.BaeI and pVSΔ2.PB40.BaeI are made to design the fiber genes, whose products would incorporate the ligands within the HI loop of the knob domain. Of note, these two plasmids are the derivatives of the pVS.PB10.BaeI and pVS.PB40.BaeI (4), respectively, each containing linker-encoding sequences that flank the site of the ligand insertion. The length of the linkers within the pVS.PB10.BaeI-encoded fibers is 10 amino acid (aa), while the fibers encoded by pVS.PB10.BaeI incorporate 20 aa-long linkers. Each of these vectors contains a unique recognition site for the restriction endonuclease BaeI, which allows for a "seamless" fusion of the ligand with the fiber protein (4).

2. Derivation of expression vectors encoding the ligand-modified fibers. At the next step we selected those peptides (from the previously published), which would allow us to maximize the chances for the positive outcome of the project. The problem we were facing in making this judgment was that while one of the papers, which we based our strategy on, described only two targeting peptides (1), the other publication listed a family of twenty-five closely related peptide sequences with only one of them being functionally tested (2). Obviously, testing all of these twenty-five peptides in this project would be rather unrealistic. Thus, in addition to using the peptides described in Ref. 1, *SMSIARL* and *VSFLEYR*, we also decided to include in our study two of the peptides described in Ref. 2, *GRRAGGS* and *AGGVAGG*. The rationale for selecting *GRRAGGS* was obvious – that was the only peptide fully characterized in the published study (2). Peptide *AGGVAGG* was also included in our project because its sequence contains two copies of the consensus tripeptide *AGG* motif that is seen in all of the prostate vasculature-specific peptides identified by Arap *et al.* (2). Thus, we reasoned that having this duplication of the core binding motif in one ligand sequence would increase the affinity of the resultant fiber protein and, subsequently, Ad vector for the target receptor, IL-11Rα.

Additional considerations that were taken into account were warranted by the fact that peptide ligands identified by the phage display technology (1, 2) are constrained by the flanking cysteine (Cys) residues. These Cys residues are introduced into the design of the phage library to stabilize the target-binding configuration of the otherwise flexible peptide ligands via the formation of a disulfide bond. While this constraining approach apparently works with the phage-displayed peptides, it is suitability for Ad targeting is not immediately apparent. This is because all of the Ad capsid proteins are translocated to the nucleus shortly after translation and, thus, never become available to the disulfide isomerases, the ER-localized enzymes that facilitate the formation of the disulfide bonds. Although we chose to use the constrained configurations of each of the four selected peptides (above), we also decided to include in our study the Cys-free versions of those peptides, whereby the Cys residues were either deleted, or replaced with residues of valine (Val) and alanine (Ala). This Val/Ala combination was previously found to most closely mimic the geometry of the Cys-Cys pair (11). Thus, each of the four selected peptide ligands was included in the study in three different configurations, thereby yielding twelve candidate ligands. The sequences of all peptide ligands used in this work are summarized in Table 1.

According to this experimental plan, DNA sequences encoding the selected peptides were assembled using oligonucleotide duplexes and cloned into Bael-cut expression vectors. Upon transformation of *E.coli*, colonies containing recombinant plasmids were identified by PCR using the insert-specific primers. Subsequently, the correct structure of these plasmids was verified by sequencing. In summary, a total of thirty-six plasmid vectors expressing the modified fiber genes have been designed.

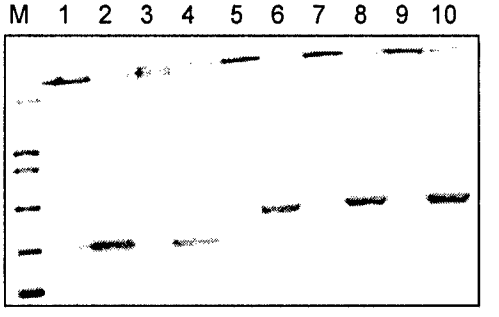
3. Expression and characterization of modified fiber proteins. The goal of this part of work was to make sure that the ligand-modified fibers can be efficiently expressed in human cells and, most importantly, that the resultant proteins retain the capacity of the wild type Ad5 fiber to form homotrimers. This trimerization ability is a key structural requirement for a functional fiber, because previous studies have shown that monomeric fibers cannot be incorporated into Ad capsids (7).

This task has been accomplished by transfecting 293T cells with the fiber-expressing vectors and then analyzing the resultant products present in the cell lysates using Western blotting. Of note, each of the tested lysates was loaded on the gel in both the fully denatured (boiled for 5 min) or non-denatured form. Comparison of the patterns produced by these samples allowed for identification of those fiber configurations that failed to form trimers under non-denaturing conditions and thus had to be eliminated from the subsequent work. Samples of the lysed cells expressing the wild type Ad5 fiber were used as a positive control of trimerization. A typical result of such a test is shown in Fig. 1.

Figure 1. Western blot analysis of the transiently expressed fiber proteins. The lysates of the transfected cells expressing the following fiber species were analyzed:

1. wild type Ad5 fiber (native)
2. wild type Ad5 fiber (denatured)
3. double-mutated Ad5 fiber (FΔ2) (native)
4. double-mutated Ad5 fiber (FΔ2) (denatured)
5. FΔ2.PB40-*GRRAGGS* (native)
6. FΔ2.PB40-*GRRAGGS* (denatured)
7. FΔ2.PB40-*CGRRAGGSC* (native)
8. FΔ2.PB40-*CGRRAGGSC* (denatured)
9. FΔ2.PB40-*VGRRAGGSA* (native)
10. FΔ2.PB40-*VGRRAGGSA* (denatured)

M Protein mol. mass standard



In summary, our work showed that, as a general trend, the incorporation of targeting peptides into the fiber had destabilizing effect on the resultant protein with some of the modified fiber proteins showing more efficient trimerization, that the others. The most important result of this work is, however, that we have identified structurally stable fiber configurations for the majority of the peptide ligands chosen. These protein species are identified by the "good" score in Table 1. The fiber fusions with only three peptides (*VSMSIARLA, CVSFLEYRC, and VVSFLEYRA*) scored as "fair".

Table 1. Trimerization by various peptide-modified fiber proteins. Scores "good", "fair" and "good" show the efficacy, with which a given fiber-ligand fusion trimerizes.

Peptide	Vector (site of ligand insertion)		
	pVSΔ2.PB10.BaeI (extended HI loop)	pVSΔ2.PB40.BaeI (extended HI loop)	pVSΔ2.FcBaeI (carboxy terminus)
SMSIARL	fair	fair	good
CSMSIARLC	good	fair	fair
VSMSIARLA	fair	fair	fair
VSFLEYR	fair	good	good
CVSFLEYRC	poor	fair	fair
VVSFLEYRA	poor	fair	fair
GRRAGGS	good	good	good
CGRRAGGSC	good	good	good
VGRRAGGSA	good	good	good
AGGVAGG	good	poor	good
CAGGVAGGC	good	good	good
VAGGVAGGA	good	good	good

Task 2.

Due to our recent relocation from the University of Alabama at Birmingham to the University of Texas MD Anderson Cancer Center, we had to spend a lot of time transferring this Award from one institution to another and starting the work at new place. In fact, the funds provided by this Award were not available to us until April 2004, at which time the work started. Because of this substantial delay, the work is presently about three months behind the schedule (Statement of Work), and the experiments described in Task 2 of the SOW are yet to commence. It is our intention to continue the work along the timeline presented in the SOW and make any adjustments, if necessary, at the end of the Year 2 of the project.

KEY RESEARCH ACCOMPLISHMENTS:

- A panel of thirty-six plasmid vectors encoding the fiber-ligand fusion proteins has been generated
- All candidate fiber-ligand proteins have been transiently expressed in cultured human cells and their ability to self-trimerize was tested by Western blotting
- Lead fiber-ligand constructs have been identified

REPORTABLE OUTCOMES: none at this early point.

CONCLUSIONS:

During the reported period the work progressed along the lines of the workplan outlined in the Statement of Work except for the delay caused by our relocation from one research institution to another.

We have been able to generate, express and characterize the products of all genetic constructs we originally planned to design. The fact that the trimerization profiles of some of the generated fiber chimeras are less than complete is nothing unheard of: similar results have been reported by others REF and are a direct consequence of structural conflict frequently seen in fiber-peptide chimeras. On the positive side and most importantly, the number of successful fiber constructs generated in Year 1 of the project is more than sufficient to support the work proposed for Years 2 and 3.

This study remains to be unique in a sense that we are not aware of any recent efforts by the competing groups, which would be aimed at the design of the Ad vectors similar to those proposed herein.

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